REMARKS

The Office Action dated November 6, 2001 presents the examination of claims 2-10, 12 and 13. In a Preliminary Amendment filed on August 15, 2001, claim 13 was amended to meet the rejections of the Examiner. However, the Examiner balks at entering the amended claim 13 in the outstanding Office Action because the claim was erroneously referred to as "Amended" rather than "Twice Amended" in the Preliminary Amendment. In order to clarify that claim 13 was twice amended, claim 13 is re-submitted herein and correctly referred to as "Twice Amended." The reference to claim 13 as "Amended" rather then "Twice Amended" occurred purely by error and the resubmission thereof does not present any new issues. In other words, no change to the body of claim 13 is presented herein. Entry of the claim is respectfully requested. No new matter is inserted into the application.

Rejection under 35 U.S.C. § 112, first paragraph

The Examiner rejects claims 2-10, 12, and 13 under 35 U.S.C. § 112, first paragraph for allegedly not being enabled by the specification. Applicant respectfully traverses. Reconsideration of the claims and withdrawal of the instant rejection are respectfully requested.

Specifically, the Examiner maintains that it is necessary to set a detection limit in claim 13. The Examiner also rejects claim 10, which is drawn to a kit, because the method of claim 13 is allegedly non-enabled.

Claim 13 recites that the target DNA and sample DNA exist in a ratio of A/B. However, there is no numerical lower limit on the amount of target nucleic acid that may be utilized. Instead, Applicant has added functional language to claim 13 stating that the mutated or polymorphic target DNA is present in an *amplifiable amount*, so that any amount of DNA that could not be amplified because the amount is too small and would be excluded from the scope of the claim. The use of "amplifiable amount" is similar to "effective amount" held to be definite by the CCPA on several occasions. See, In re Halleck, 422 F2d 911 (CCPA 1970); In re Watson, 517 F2d 465 (CCPA 1975); In re Caldwell, 319 F2d 254 (CCPA 1963). In these cases, the Federal Court has held that the term is not objectionable where the amount is not critical, or where one skilled in the art can determine from the disclosure what an effective amount is, or when the effect achieved is recited.

In the present case, it is known in the art that, in the method of PCR, the labeled standard DNA enables the reliable detection of the target DNA even if the amount of target DNA corresponds to one molecule. A simple search of relevant

literature also illustrates that the ability of PCR to amplify low amounts of target DNA. As evidence thereof, the Examiner's attention is drawn to Li et al. Amplification and analysis of DNA sequence in single human sperm and dipoid cells (*Nature*, Vol. 335 29 September 1988), wherein amounts as low as 3.3×10^{-9} fmol of DNA was amplified. (See Exhibit 1). Further, Mastrangelo et al. (*Surv. Ophthalmol.* 1997 Jan-Feb; 41(4):331-40) states, "The Polymerase Chain Reaction (PCR) is a highly innovative technique which allows for the generation of large amounts of DNA starting from minute quantities obtained from the blood or tissue of a patient." (See Exhibit 2).

Nonetheless, the Examiner maintains his assertion that the amount of target DNA may be so small that it cannot be detected. In support thereof, the Examiner points to page 11, and asserts that a DNA of 1% cannot be detected. However, the Examiner fails to appreciate the disclosure in page 11. Specifically, the passage pointed out by the Examiner states,

> However, when the content of the target DNA which is the same as the labeled standard DNA is only 1%, and the assay is conducted at the excessiveness of the sample DNA of 10, the dilution of the labeled standard DNA is 10/11, and the labeled standard DNA is not substantially diluted. Therefore, the DNA whose content is 1% is not detected.

(See Exhibit 3). This description merely indicates that when the content of the target DNA is the same as the labeled standard DNA,

specifically 1%, then the excessiveness of the sample should be set to "100" rather than "10" in order to detect the target DNA. Thus, this passage unequivocally does *not* state that a DNA of 1% cannot be detected. The Examiner is directed to page 14 of the specification, wherein it is stated that DNA in an amount of less than 1 μg is amplifiable:

> The specimen may preferably contain the DNA in a total amount of about 1 to 100 μg although the DNA in an amount of less than 1 μg is well amplifiable.

(See Exhibit 4).

The fact that less than 1 μg of target DNA can be detected is also illustrated by the mathematical configuration of the genome. For example, the mammalian genome size is 3.3×10^9 (bp). (See Exhibit 5). Since the molecular weight of one basepair is 660, the molecular weight of the mammalian genome is approximately 2.18×10^{12} ($3.3 \times 10^9 \times 660 = 2.18 \times 10^{12}$). When the molecular weight of the mammalian genome is divided by Avogadro's constant, 6.02×10^{23}, the weight in one molecule of the mammalian genome corresponds to 3.6×10^{-12} (g), or 3.6×10^{-6} μg. Obviously, this number is much lower than 1 μg.

In summary, the above-cited references clearly support that an amount of less than 1 μg can be detected sufficiently in a general PCR method. One of ordinary skill in the art would therefore understand that an amount of less than 1 μg of target DNA can be

detected sufficiently even if the lower limit of the target DNA is not specifically set forth in claim 13.

Thus, Applicant respectfully submits that the rejection is improper and should be withdrawn.

Summary

Applicant respectfully submits that the above amendments and remarks alleviate the Examiner's outstanding rejections such that the present invention is in a condition for allowance. Favorable action and early allowance of the claims are respectfully requested.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact Kristi L. Rupert, Ph.D. (Reg. No. 45,702) at 703/205-8000 in the Washington Metropolitan Area.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees

required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

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GMM/KLR
0171-0613P

Attachment: Version with Markings to Show Changes Made
Exhibit 1 - Li et al.
Exhibit 2 - Mastrangelo et al.
Exhibit 3 - Page 11 of specification
Exhibit 4 - Page 14 of specification
Exhibit 5 - Benjamin Lewin Genes V

(Rev. 02/12/01)

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Claims:

The claims have been amended as follows:

Claim 13. (Twice Amended) A nucleic acid assay process for identifying and/or quantifying a mutation or polymorphism in a double stranded sample DNA prepared by amplification of a particular region of an analyte nucleic acid which is present in a specimen, comprising the steps of:

providing labeled standard DNA having a nucleotide sequence the same as a mutated or polymorphic target DNA of interest, wherein said labeled standard DNA comprises a double stranded nucleic acid having a site capable of binding to a solid support on one strand and a detectable label on the other strand;

amplifying said [a] particular region of said [an] analyte nucleic acid which is present in said [a] specimen to prepare said [a] double stranded sample DNA, for competitive hybridization wherein said sample DNA comprises both wild-type and mutated or polymorphic target DNA in an amplifiable amount;

selecting a detection limit for said mutated or polymorphic target DNA, wherein when the detection limit for the target DNA present in said sample DNA is A/B, the excessiveness of said sample

DNA is at least B/A, and wherein A/B is the fractional equivalent of the percentage of target DNA content in the sample DNA;

adding an excessive amount of said sample DNA to said labeled standard DNA, to allow competitive hybridization to take place between said target DNA and labeled standard DNA under conditions which allow for hybridization [rehybridization] of at least some of said labeled standard DNA and under conditions wherein non-target sample DNA does not hybridize with said labeled standard DNA, wherein the excessiveness of said sample DNA added to said labeled standard DNA in the competitive hybridization is selected in accordance with the pre-selected detection limit,

detecting the hybridized [rehybridized] labeled standard DNA by utilizing said detectable label and said site capable of binding to a solid support; and

evaluating the degree of exchange that occurred during competitive hybridization of the complementary strands between said sample DNA and said labeled standard DNA.

In a future paper, we will examine strategies to search for the stringiness of an anisotropy pattern in more detail by running Monte Carlo experiments on our anisotropy map. We will also use Monte Carlo experiments to determine the proper limits on $G\mu/c^2$ from small-angular-scale experiments. Finally, we will also attempt a more quantitative estimate of other anisotropies predicted by cosmic string models, which might interfere with the stringy nature of the anisotropies discussed here.

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Amplification and analysis of DNA sequences in single human sperm and diploid cells

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The use of the polymerase chain reaction for analysing DNA sequences in individual diploid cells and human sperm shows that two genetic loci can be co-amplified from a single sperm, which may allow the analysis of previously inaccessible genetic phenomena.

THE construction of genetic maps in higher organisms requires analysis of the progeny of selected matings or calculation of linkage relationships by pedigree analysis. In humans only the latter is possible. Using restriction fragment length polymorphisms (RFLPs), there has been significant progress towards the construction of a human linkage map (for review see ref. 1). To locate genes with known phenotypic effects relative to RFLP markers there has been a concerted effort to establish a panel of genetic markers at about 10 cM (1 cM = 1% recombination) intervals so that no gene will be further than 5 cM away from an RFLP (ref. 2). Pedigree analysis is thought to be able to measure genetic distances of about 1 cM encompassing about 1,000 kilobases (kb) of DNA with statistical reliability. The analysis of smaller distances requires such a large number of individuals from informative familes that it is impractical. If the genotype of large numbers of individual sperm could be determined, the measurement of genetic recombination over shorter physical distances could be accomplished at a resolution far greater than that currently possible and without family studies. We have therefore attempted to define the conditions required to analyse DNA sequences in single cells using the polymerase chain reaction (PCR, refs 3 and 4). We studied single diploid cells and then single sperm from an individual heterozygous at two genetic loci found on non-homologous chromosomes. For each locus we determined which of the two alleles were present in any one sperm and analysed the independent assortment of the alleles at a single locus and the independent segregation of genes on non-homologous chromosomes.

Analysis in single diploid cells

We studied the human β-globin gene locus in individual diploid cells from two tissue culture cell lines. One was derived from a homozygous individual for the sickle-cell mutation at codon six (β^S); the other was homozygous for the normal β^A allele. PCR primers that amplify a β-globin fragment containing codon six and allele-specific oligonucleotide probes (ASO) which can distinguish between these two alleles have already been described[3,5]. We co-cultivated the cells homozygous for β^A and cells homozygous for β^S in the same tissue culture flask for several days. Individual cells from this mixture were drawn into a thin plastic pipette during observation under a phase-contrast microscope. Each individual cell was delivered into a PCR tube containing a lysis solution and, after incubation, PCR buffer containing deoxyribonucleotides, *Taq* DNA polymerase[6] and a set of PCR primers that amplify the informative region of the globin gene was added. After DNA denaturation 50 cycles of amplification were performed[6]. Aliquots from each sample of amplified product were hybridized separately with the β^A and β^S probes after fixation to nylon membranes[4,7]. The results of this co-cultivation experiment are shown in Fig. 1. Out of the 37 cells analysed, 84% hybridized with only one of the two allele-specific probes; 19 with the β^A probe and 12 with the β^S probe. None of the 12 control tubes, which received water instead of a cell, was positive, indicating that DNA contamination was insignificant. No sample hybridized with both probes, indicating that a single cell only was introduced into each tube and that DNA from lysed cells present in the co-cultivation mixture did not adhere to individual cells.

The amount of amplified β-globin gene product produced by PCR of a single cell was determined by comparing the intensity of the hybridization signal obtained with that from known amounts of plasmid DNA carrying the globin gene spotted on the same filter (Fig. 1). We estimate that, starting with a diploid amount of globin DNA (3.3×10^{-9} fmol), between 5 and 500 fmol of PCR product was produced in 50 cycles. This is equivalent to an average amplification ratio of 7.6×10^{10} with

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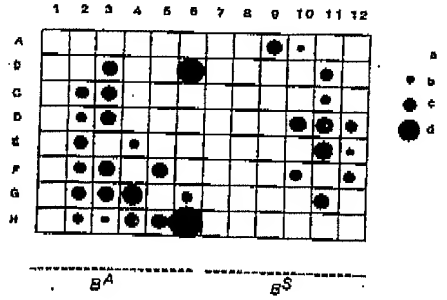

Fig. 1 PCR analysis of the β-globin gene in individual tissue culture cells. Each of two aliquots from the PCR products of a single cell were placed in the same row of the dot blot apparatus separated by six columns. Half of the filter was hybridized to the β^A ASO and the other to the β^S ASO. 1A–1H (7A–7H) and 2A, 2B (8A, 8B) were water blanks. 6H and 12H are aliquots of PCR product of purified β^A DNA. One, 3, 10 and 30 fmol of β^S gene containing plasmid were dotted as hybridization standards at positions a, b, c and d respectively. The remaining samples were individual tissue culture cells. After washing three times single cells were selected from a cell suspension ($1–3\times10^6$ ml^{-1}) with a Zeiss phase-contrast microscope at ×100 using a plastic needle with a 0.1-mm-diameter opening made by pulling a flamed disposable 1-ml-graduated plastic pipet. Each single cell sample was delivered into a 0.5 ml plastic microfuge tube containing 10 µl autoclaved distilled water. The cells were lysed using a slight modification of a published sperm lysis procedure[10]. The sample was adjusted to a final volume of 20 µl containing 1 × PCR reaction buffer (ref. 6; 50 mM KCl, 10 mM Tris-HCl *p*H 8.3, 2.5 mM $MgCl_2$, 0.1 mg ml^{-1} gelatin), 0.05 mg ml^{-1} proteinase K, 20 mM DTT, and 1.7 µM SDS. After one hour at 37 °C, samples were heated to 85 °C, and suspended in 100 µl PCR reaction mix containing 1 × PCR buffer, 1 µM each oligonucleotide PCR primer, 187.5 µM each of dATP, dCTP, dGTP and dTTP, 100 ng *E. coli.* DNA, 2 units of *Thermus auquaticus* thermostable DNA polymerase (Perkin Elmer-Cetus Instruments), and 60 µl mineral oil to prevent evaporation. The PCR reactions were carried out on a DNA Thermal Cycler (Perkin Elmer-Cetus Instruments). After heating at 95 °C for 10 min to ensure DNA denaturation, each cycle of PCR consisted of incubation at 95 °C for 15 s, 15 s incubation at 54 °C and a 1 min incubation at 72 °C. After fifty cycles of PCR, dot blot analysis of 20 µl samples of the PCR reaction were carried out using β^S- and β^A-allele specific probes[5].

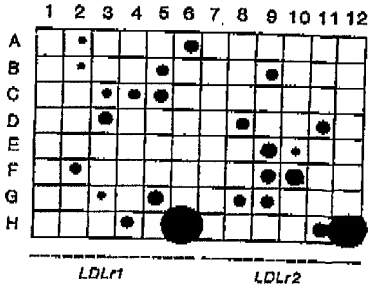

Fig. 2 PCR analysis of the LDL receptor locus of individual human sperm. 1A–1H (7A–7H) are water blanks. 6H and 12H are aliquots of amplified DNA from an *LDLr*1/*LDLr*2 heterozygote. The remaining samples are from individual sperm. The organization of the samples on the dot blot is as in Fig. 1. Sperm were purified from semen by a modification of a published procedure[10]: 0.5 ml semen was mixed with 3 ml of 40% sucrose. The mixture was applied to the top of a sucrose step gradient made by adding 3.5 ml 90%, 70% and 50% (w/v) sucrose successively to a 15-ml graduated plastic tube (Falcon 2095). The sample was spun at 14,500g for two hours at room temperature. 0.5 ml of the interface between 70% and 90% sucrose was collected and applied to an identical sucrose gradient. This was repeated twice more. Single sperm were isolated in the same way as individual diploid cells using a sperm suspension at a concentration of 1×10^5 sperm ml^{-1}. The sequence of the two primers used for PCR of the *LDLr* locus were 5'AGTGCCAACCGCCTCACAGG3' and 5'CCTCTCACACCAGTTCACTC3'. The ASO for the *LDLr*1 allele had the sequence 5'AGGATATGGTCCTCTTCCA3' whereas the *LDLr*2 ASO had the sequence 5'TGGAAGAGAACCATATCCT3'. The PCR and dot blot analysis were performed as in Fig. 1 except that the final washes of the filters hybridized with the *LDLr* probes (and *HLA DQA* probes, see Fig. 3) were at 56 °C.

an average efficiency per cycle of 65%. The precise extent of amplification may have been slightly lower if these cell lines contained more than two copies of chromosome 11. Considering the extent of amplification, elimination of all sources of possible contamination is critical to the success of these experiments.

Analysis in single human sperm

The genotype of single sperm derived from an individual heterozygous at the gene coding for the low density lipoprotein receptor (the *LDLr* gene), which has been localized to chromosome 19 (ref. 8), was analysed next. We adapted the detection of an *LDLr* polymorphism[9] to PCR and ASO analysis using DNA sequence information (D. Russell, unpublished data), with the PCR primers and probes shown in the Fig. 2 legend. The size of the expected PCR product was 254 base pairs (bp).

Sperm were purified from a semen sample by centrifugation through a sucrose step gradient[10] and stored for 8 months at −20 °C. Individual sperm were drawn into a fine plastic needle under microscopic observation and delivered to a tube for lysis and amplification. In a series of experiments we analysed the *LDLr* genotypes in 80 indiyidual sperm: typical results from one such experiment are shown in Fig. 2. Altogether 55% of the sperm gave a hybridization signal. Twenty-two carried one allele; 21 the other. Only one sample was positive with both probes. Sixteen additional control tubes which received all of the reagents but no sperm did not give any hybridization signal. The distribution of the two amplified alleles obeyed Mendel's law of independent segregation, indicating that the PCR reactions were initiated with a single meiotic product and that no contaminating diploid DNA sequences were present.

Independent assortment of chromosomes

We attempted to amplify simultaneously DNA sequences at two different loci on non-homologous chromosomes in a single sperm. Our sperm donor was heterozygous at the *HLA DQ-α* locus (*DQA*) on chromosome six, as well as at the *LDLr* gene. Primers for amplification of the first gene and probes to distinguish between allelic variants have been described previously[5,11]. The predicted size of the PCR product was 242 bp. Initial experiments in which primers for both loci were present throughout the entire amplification experiment were unsuccessful, so we performed only the first 20 amplification cycles in the presence of both primer pairs. After this primary amplification, 1/50 of the reaction mixture was placed in a tube and diluted with a PCR solution containing the *HLA* primers only and another aliquot was diluted with a PCR mix containing the *LDLr* primers only. After an additional 45 cycles of amplification, part of each secondary reaction was hybridized to either of the two ASOs for that locus. A total of 150 individual sperm were analysed, in a series of such experiments (Table 1; representative data are shown in Fig. 3). Twenty-seven samples did not exhibit amplification of either locus, but we did detect hybridization signals in 123 samples (82%). In nine of these samples, two alleles from at least one of the two loci were

Fig. 3 PCR analysis of individual sperm simultaneously amplified with *HLA DQA* and *LDLr* primers and analysed with allele-specific probes for the *DQA* alleles 1 and 2 (*DQA1*, *DQA2*) and *LDLr* alleles *1* and *2* (*LDLr1*, *LDLr2*). Water blanks are in positions 19–21. Examples of sperm where one allele at each locus is amplified are shown in positions 2, 5, 6, 8, 10, 11, 13, 14 and 16. The remaining samples can be divided into those that amplified more than one allele at a locus (position 4), only one allele at one locus (positions 1, 3, 7, 9, 12, 17, 18) or neither allele (position 15). During the first 20 cycles both loci were amplified simultaneously with both sets of primers present at 1 μM. A 2 μl aliquot from each reaction was then added to each of two tubes with 100 μl fresh PCR reaction buffer containing only one of the primer pairs. Forty-five additional PCR cycles were then performed.

detected and these samples were excluded from further analysis. These samples may have contained two sperm of different genotypes. On the other hand these nine samples might have resulted from non-disjunction events. The analysis of only two loci cannot distinguish between these possibilities.

Among the remaining 114 sperm, 96 could be typed at the *LDLr* locus with 45 having *LDLr*1 and 51 having *LDLr*2. The two alleles segregate in the expected 1:1 ratio (Chi square = 0.375, $0.75 > P > 0.5$, 1 degree of freedom). Eighty-eight could be typed at the *DQA* locus: 53 had the *DQA*1 allele, 35 the *DQA*2. The segregation of the *DQA* alleles with the expected 1:1 ratio is at the borderline of statistical significance (Chi square = 3.68, $0.1 > P > 0.05$, 1 degree of freedom). This could represent a statistical fluctuation, unequal amplification of the two *DQA* alleles resulting from base mismatches between the PCR primers and the *DQA* DNA sequence of our particular donor due to the extensive polymorphism of this locus, or some unusual genetic phenomenon such as segregation distortion.

Seventy of the 114 sperm (61%) that gave hybridization signals could be typed at both loci. The independent segregation of chromosome six and chromosome 19 should result in the equally frequent occurrence of the four possible gametes: *DQA*1, *LDLr*1; *DQA*1, *LDLr*2; *DQA*2, *LDLr*1; *DQA*2, *LDLr*2. We actually observed 21, 18, 14 and 17 of each type respectively; the difference is not statistically significant (Chi square = 1.43, $0.75 > P > 0.5$, 3 degrees of freedom). These results show that we can reliably and with reasonable efficiency simultaneously determine the genotype of individual sperm at two distinct genetic loci.

Among the 114 samples that gave a hybridization signal, 18 showed one of the two *DQA* alleles but an *LDLr* product could not be detected. Twenty-six sperm showed one of the two *LDLr* alleles but no amplification of the *HLA* locus. These sperm could be nullosomic for one of the two chromosomes we studied due to a non-disjunction event. There may also have been a failure to amplify one of the two loci. Because we only looked at a single locus on each chromosome we cannot distinguish between these possibilities.

The relative frequency of successful amplification of the *LDLr* and *DQA* loci in single sperm is approximately the same. Among 141 samples, 88 amplified *DQA* and 96 amplified *LDLr*. We are unable to calculate accurately the absolute frequency of success because we cannot be sure that every one of the 141 samples did in fact contain a sperm. As a result, we cannot use our data to calculate accurately the expected frequency of samples with a single allele amplified at both loci, at only one locus or at neither locus assuming that amplification at each locus is an independent event. Amplification of both loci may not be independent events if sperm lysis is not uniform from sample to sample. Thus the assessibility of one of the target DNA molecules to the PCR reagents might be positively correlated with the accessibility of the other. Improving the absolute rate of successful amplification of single sperm may depend upon improving lysis procedures, thereby enhancing target DNA accessibility.

Discussion

The analysis of the genotype of single sperm at the DNA level is a unique tool for the study of problems in human genetics considered intractable using current approaches. To date there have been no practical methods for accurate measurement of genetic distances of less than 1 cM. A significant advantage of the approach described here is that a large number of meiotic products can be examined from a single individual allowing determination of the recombination frequency between genetic markers which are physically very close. In conjunction with gel electrophoresis procedures for very large DNA fragments and chromosome-walking data it should be possible to measure the frequency of recombination between genetic markers whose physical distance apart is known precisely. This would allow the analysis of recombination frequency as a function of physical distance and a test of the conventionally accepted value of one per cent recombination per million base pairs[12] for specific chromosomal regions. Because it should be possible to obtain statistically significant data on recombination frequencies from a single individual, it should also be possible to determine for the first time whether different individuals have the same or different rates of recombination for the same interval.

The ability to measure recombination over short physical distances will be especially useful in the study of recombination hot spots. The effect that a hot spot can have on recombination between flanking markers depends upon how far away the flanking marker loci are from the ends of the hot spot. Thus a 1-kb DNA segment with a recombination potential which is 10-fold greater than normal DNA will have very little effect on recombination measured between markers that are 500 kb to either side of the hot spot. Pedigree analysis cannot measure recombination over the short intervals typical of many of the hot spot regions that have been deduced from population genetics data[13–15], given the number of informative families required and the effort involved in obtaining the data. With PCR, we can envisage typing as many as 500 meiotic products in a week. Decreasing the number of samples containing two sperm and increasing the efficiency of amplification of both loci simultaneously will be required for the highest resolution

Table 1 Amplification of sequences at two different loci

Total number of sperm examined	150
No signal	27
*DQA*1, *LDLr*1	21
*DQA*1, *LDLr*2	18
*DQA*2, *LDLr*1	14
*DQA*2, *LDLr*2	17
*DQA*1	14
*DQA*2	4
*LDLr*1	10
*LDLr*2	16
*DQA*1, *LDLr*1, *LDLr*2	2
*DQA*2, *LDLr*1, *LDLr*2	1
*DQA*1, *DQA*2	1
*LDLr*1, *LDLr*2	2
*DQA*1, *DQA*2, *LDLr*1, *LDLr*2	3
Controls	32
No signal	29
*LDLr*1	2
*LDLr*2	1

studies. In linkage experiments a fraction of the samples that contain two sperm and which have amplified only one of the two alleles at each locus will generate 'false recombinants'. We expect that the frequency of false recombinants can be minimized by careful attention to sperm isolation and the enhancement of sperm lysis and amplification efficiency. If very large numbers of sperm could be analysed with great reliability, some mutational events which cannot be analysed by conventional methods might eventually be studied.

Our ability to haplotype sperm at the DNA level will provide a fundamentally new approach to studying human recombination and may also be useful in determining the physical order of DNA polymorphisms which are so tightly linked that they cannot be resolved by additional family analysis. This may be especially significant in the case of random RFLPs tightly linked to disease-causing loci. The genetic distances between the random RFLPs could be accurately determined and the RFLPs ordered with respect to one another by three point crosses, provided that simultaneous amplification of 3 marker loci could be made efficient enough for single sperm experiments. Such fine structure maps might be of great value in attempts to locate the disease-causing locus itself. Of course because sperm do not exhibit disease phenotypes, an unknown disease locus cannot be directly mapped relative to polymorphisms in this way.

The analysis of single sperm in species that cannot be extensively bred or have exceptionally long generation times may be the only practical way of making genetic maps for these species.

One immediate practical application of the analysis of individual sperm is in the area of forensic medicine. *HLA* typing for paternity determinations or identification of criminals is often hampered by the inability to determine the haplotype of the suspected individuals because this would require the analysis of close relatives. *HLA* analysis of individual sperm from a suspect would allow the linkage phase of the *HLA* markers to be unambiguously determined and thus increase the probability of inclusion or exclusion.

Finally, the ability to study DNA sequences in individual diploid cells will make it possible to study cell-to-cell variation in developmental processes involving DNA rearrangements or other genetic alterations. It is also likely that analysis of messenger RNAs in single cells would be possible if efficient reverse transcription could be carried out before PCR was initiated. Prenatal diagnosis on a single cell derived from a preimplantation embryo resulting from *in vitro* fertilization is also conceivable.

We thank L. Goda, D. Spasic and C. Levenson for synthesis of the DQA primers and probes. U.B.G. was supported by a Knut and Alice Wallenberg Foundation Fellowship. This work was supported in part by a grant from the National Institute of General Medical Sciences (N.A.).

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LETTERS TO NATURE

No cometesimals in the inner Solar System

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Ultraviolet measurements made by Voyager 2, apparently showing a rapid decrease in hydrogen Lyman-α emission with distance from the Sun, were taken by Donahue *et al.*[1] as evidence for a source of atomic hydrogen in the very local interstellar medium (VLISM). The suggested source[1] is a class of small comets, at solar distances of ~1 AU, that produce atomic hydrogen as their icy mantle is evaporated. This claim has been adduced as evidence for a theory[2] that the Earth is subject to a large influx of cometary material, significantly affecting atmospheric evolution. Here we analyse again the Voyager 2 data, and show that no source of hydrogen in the VLSIM is required other than the inflow of neutral atoms; the original analysis was apparently flawed by an erroneous transcription of tabulated data (T. M. Donahue, personal communication).

Although Donahue *et al.*[1] claimed to find a cometary source of hydrogen, the estimated flux was ~seven orders of magnitude smaller than required by the theory of Frank *et al.*[2]. But by postulating a different and more numerous kind of comet, Frank *et al.* have claimed[3] that the Voyager data, the most important direct evidence for small comets and the crucial data in obtaining limits on sources of volatile compounds such as H_2O, can be made to agree with their theory.

The observations in question are obtained with Voyager 2 shortly after launch[1]. Figure 1 shows the observing geometry looking down on the north pole of the Solar System. A number of observations, in a direction approximately downstream with respect to the inflowing VLISM neutral gas, were obtained with the spacecraft located near 1 AU. Further measurements were obtained with the spacecraft located at $r_0 \sim 1.3$ AU and beyond, with all observations approximately downstream as shown in Fig. 1. The analysis of these data by Donahue *et al.*[1] indicated that the measurements near 1 AU in a direction almost tangential to the Earth's orbit demonstrated intensities in excess of a normal VLISM model. The excess near 1 AU was attributed to the influx of cometesimals. We argue that the stronger signal obtained near 1 AU is simply a consequence of observing geometry.

The search for a local source of hydrogen in the Voyager data was limited by Donahue *et al.*[1] to observations in the VLISM downstream direction (Fig. 1). The observations obtained with the spacecraft positioned near 1 AU were necessarily at a high angle ($\beta \approx 90°$; see Fig. 1) to the antisolar direction because the spacecraft was positioned in the vicinity of 0° right ascension (α) (Fig. 1). Later observations with the spacecraft positioned near 2 AU were obtained with a viewing direction more closely aligned with the antisolar direction. A non-negligible fraction

EXHIBIT 2

1: Surv Ophthalmol 1997 Jan-Feb;41(4):331-40 Related Articles, Books, LinkOut

The polymerase chain reaction (PCR) in the routine genetic characterization of retinoblastoma: a tool for the clinical laboratory.

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The Polymerase Chain Reaction (PCR) is a highly innovative technique which allows for the generation of large amounts of DNA starting from minute quantities obtained from the blood or tissue of a patient. With the increasing knowledge concerning the structure of the human genome and the potential to amplify specific segments of DNA by the PCR technique, the molecular genetic characterization of many ocular disorders has been greatly facilitated. This is particularly true of retinoblastoma (RB) where the causative gene, RB1, gene has been identified and characterized. Using PCR technique, specific sequences of the RB1 gene can be amplified and analyzed to precisely define the genetic mutation in an affected individual. In addition, this technique can also be applied in order to characterize the genetic defect within the tumor itself. In this report we illustrate the use of the PCR technique in the genetic characterization of the RB1 gene and its application to the study of RB. These techniques are applicable even in a small clinical laboratory and can be extended to a number of ophthalmic disorders.

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EXHIBIT 3

solid support to measure the label intensity to thereby measure the rehybridized labeled standard DNA. The presence/absence of the nucleic acid fragment including the base sequence the same as the labeled standard DNA is thereby determined.

However, when the content of the target DNA which is the same as the labeled standard DNA is only 1%, and the assay is conducted at the excessiveness of the sample DNA of 10, the dilution of the labeled standard DNA is 10/11, and the labeled standard DNA is not substantially diluted. Therefore, the DNA whose content is 1% is not detected.

In view of such situation, in the present invention, a detection limit is preliminarily selected for the target DNA which is the same as the labeled standard DNA and which is present in the sample DNA. For example, when the target DNA whose content in the sample DNA is about 5% (1/20) is detected, the sample DNA1 is used at an excessiveness of, for example, 20 or more as shown in FIG. 1, and use of the DNA1 in such an amount means that 1/20 of the sample DNA1 is the target DNA whose base sequence is the same as the labeled standard DNA2. The labeled standard DNA2 is then diluted to 1/2, and the theoretical measurement is 1/2 of the initial measurement. A reliable detection of the target DNA which is the same as the labeled standard DNA2 and which is contained in the sample DNA1 is thereby realized. It should be noted that, even when the excessiveness of the sample DNA1 is 20 or more, the labeled standard DNA is theoretically not at all diluted if no DNA whose base sequence is the same as the labeled standard DNA2 is present in the sample DNA1. Therefore, the label intensity of the case wherein the content of the target DNA is 5% is 1/2 of the label intensity of the case wherein the target DNA is absent.

In a similar manner, when sample DNA of the amount 40 times in excess of the labeled standard DNA is added, and the test sample contains the target DNA including the base sequence the same as the labeled standard DNA in an amount of 2.5% (1/40), the proportion of the initial labeled

EXHIBIT 4

The term, polymorphic genes from the host as used herein designates genes exhibiting polymorphism which have no direct relationship with the cause of a disease, and such genes include genes related to HLA and blood types. Although such genes are usually located on the chromosome of the host, there are cases wherein such gene is located on mitochondria. Exemplary specimens which contain such analyte nucleic acid are pathogens such as a bacterium and a virus; blood, saliva, section of tissue lesion and the like separated from a living body; and excreta such as feces and urine. In the case of prenatal diagnosis, cells of fetus in the amniotic fluid or a part of the divided ovule in the test tube may be used for the specimen. The specimens may be preliminarily subjected to cytolysis by treating the specimen using an enzyme, heat or a surfactant, ultrasonication, or a combination thereof directly or after optional concentration by precipitation through a procedure like centrifugation. Such cytolysis is effected for the purpose of exposing the DNA from the target tissue. In practice, the cytolysis may be carried out in accordance with a known procedure such as the one described in PCR PROTOCOLS, Academic Press Inc., p14, p352 (1990) or other documents. The specimen may preferably contain the DNA in a total amount of about 1 to 100 μg although the DNA in an amount of less than 1 μg is well amplifiable.

The gene amplification process used in amplifying the analyte nucleic acid in the specimen for the preparation of the sample DNA is not limited to any particular process, and such amplification may be carried out by using gene amplification primers or by chemical synthesis or enzymatically linking chemically synthesized partial sequences. In preparing the sample DNA, the thus amplified DNA may be mass-produced by incorporating the DNA in a vector selected from plasmid vectors, phage vectors and chimeric vectors derived from a plasmid and a phage and introducing the vector in a propagatable host such as a bacterium such as Escherichia coli or Bacillus subtilis, or yeast (Saccharomyces cerevisiae).

EXHIBIT
5

BENJAMIN LEWIN

GENES V

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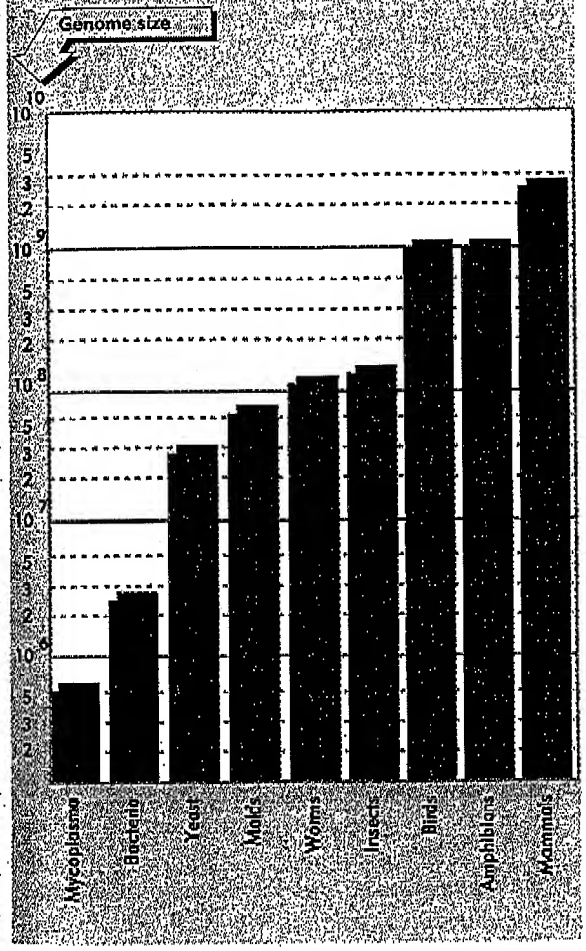
Figure 22.2

The minimum genome size found in each phylum increases from prokaryotes to mammals.



wide variations in the genome sizes within some phyla.

Plotting the *minimum* amount of DNA required for a member of each phylum suggests in Figure 22.2 that an increase in genome size is required to make more complex prokaryotes and lower eukaryotes.

The smallest genome yet identified for a living cell actually belongs to a eukaryotic, the unicellular alga *Pyrenomas salina*, at 6.6×10^5 bp. (However, these organisms may not be true eukaryotes, but could be an intermediate stage of evolution, representing a primitive presence of a nucleus and chloroplast.) Excluding these algae, mycoplasma are the smallest types of organism known, and also may be constructed from very small genomes. Thus organisms may have genome sizes only ~3× the size of a large bacteriophage (T4 is 1.7×10^5 bp). Bacteria start at ~2×10^6 bp. Unicellular eukaryotes (whose life-styles may resemble the prokaryotic) get by with genomes that are also small, although larger than those of the bacteria. Being eukaryotic *per se* does not imply a vast increase in genome size; a yeast may have a genome size of ~1.3×10^7 bp, only about twice the size of the largest bacterial genomes.

A further twofold increase in genome size is adequate to support the slime mold *D. discoideum*, able to live in either unicellular or multicellular modes. Another increase in complexity is necessary to produce the first fully multicellular organisms; the nematode worm *C. elegans* has a DNA content of 8×10^7 bp.

Climbing further along the evolutionary tree, the relationship between complexity of the

Table 22.1

The genome sizes of some common experimental animals.

Phylum	Species	Genome (bp)
Algae	*Pyrenomas salina*	6.6×10^5
Mycoplasma	*M. pneumoniae*	1.0×10^6
Bacterium	*E. coli*	4.2×10^6
Yeast	*S. cerevisiae*	1.3×10^7
Slime mold	*D. discoideum*	5.4×10^7
Nematode	*C. elegans*	8.0×10^7
Insect	*D. melanogaster*	1.4×10^8
Bird	*G. domesticus*	1.2×10^9
Amphibian	*X. laevis*	3.1×10^9
Mammal	*H. sapiens*	3.3×10^9

organism and content of DNA becomes obscure, although it is necessary to increase the genome size in order make insects, birds or amphibians, and mammals.

We can also see the steady increase in genome size with complexity in the listing in Table 22.1 of some of the most commonly analyzed organisms, although there are some real puzzles. Can the toad *X. laevis* have the same genetic complexity as man, as one might suppose naively from their common genome sizes?

In some phyla, the spread of genome sizes is narrow. Birds, reptiles, and mammals all show little variation within the phylum, with a range of genome sizes in each case about twofold. But in other cases, most notably insects, amphibians, and plants, there is a wide range of values, often more than tenfold. One wonders whether the common housefly (*Musca domestica*) with a genome size of 8.6×10^8 really has a corresponding increase in complexity over the common fruit fly, *D. melanogaster*, with its 6-times smaller genome of 1.4×10^8.

The **C-value paradox** takes its name from our inability to account for the content of the genome in terms of known functions. It expresses the existence of two puzzling features:

- There is an excess of DNA compared with the amount that could be expected to code for proteins. We can now account for much of the excess because genes are much larger than the sequences needed to code for proteins (principally because of the intervening sequences that break up a coding region into different segments). We discuss the structure of such genes, and their contribution to overall genome size, in Chapter 23.

- There are large variations in C-values between certain species whose apparent complexity does not vary much. In amphibians, the smallest genomes are just below 10^9 bp, while the largest are almost 10^{11} bp. It is hard to believe that this could reflect a 100-fold variation in the number of genes needed to specify different amphibians, so we need to account for the 'excess' DNA in some other way.

Reassociation kinetics depend on sequence complexity

The range of sizes of eukaryotic genomes raises an important question: do larger genomes contain a greater number of different genes or do they instead contain more copies of the same genes that are present in smaller genomes? If the diversity of genes increases with genome size, we should expect the amount of unique DNA sequences in the genome to increase. This will not happen if there are simply more copies of the same genes.

The general nature of the eukaryotic genome can be assessed by the kinetics with which denatured DNA reassociates. Reassociation between complementary sequences of DNA occurs by base pairing, in a reversal of the process of denaturation by which they were separated (see Figure 5.2). The technique can be extended to isolate individual DNA or RNA sequences by their ability to hybridize with a particular probe (see Chapter 21). The kinetics of the reas-

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